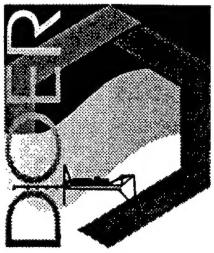

Technical Note DOER-C1
February 1998

Guidance for Performance of the H4IIE Dioxin Screening Assay

PURPOSE: This technical note provides protocols for maintenance of cell cultures and for the conduct of a biomarker-based screening assay for dioxin toxic equivalents (TCDD TEQs) in sediments and other environmental samples using the H4IIE rat hepatoma cell line as performed at the U.S. Army Engineer Waterways Experiment Station (WES). The purpose of these protocols is to provide analysts who may wish to gain the capability for performing the assay with a detailed "blueprint" of how the assay is conducted and of what is necessary in terms of technique, materials, instrumentation, and time.

BACKGROUND: The cost of gas chromatography/mass spectrometry (GC/MS) analysis for dioxins in environmental samples remains so high as to prohibit wide usage of this method for monitoring and site characterization. An alternative and supplement to GC/MS that is far less expensive (1/10th to 1/20th the cost per sample) is the use of cultured cell lines to detect and quantitate dioxin-like activity. This is possible because 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (2,3,7,8-TCDD) and structurally similar compounds bind with a receptor protein within the cell, the aryl hydrocarbon or Ah receptor (AhR). Translocation of the AhR-dioxin complex to the cell nucleus followed by binding with dioxin recognition elements (DREs) of the nuclear DNA results in the expression of detoxifying enzymes. The amount of the enzymes produced is in proportion to the concentration of dioxin. In the assay, one of these enzymes, ethoxyresorufin O-deethylase (EROD) serves as the indicator of dioxin activity. The enzyme hydrolyzes the substrate, and the amount of product formed (resorufin) is measured using a spectrofluorometer. The halogenated dibenzo-*p*-dioxins and dibenzofurans containing the 2,3,7,8-Cl substitution pattern and coplanar polychlorinated biphenyls (PCBs) with lateral substitution of at least four chlorines all bind with the AhR, but with differing intensities. The polycyclic aromatic hydrocarbons (PAHs) also bind with the AhR and may be removed in a cleanup step during sample preparation. Using this phenomenon, TCDD TEQs can now be measured in environmental samples in the low parts-per-trillion range.

The Reuber rat hepatoma H4IIE cell line was first used in assays for halogenated hydrocarbons in fish by the Food and Drug Administration (Bradlaw and Casterline 1979; Casterline et al. 1983). In recent years, it has been used extensively by the U.S. Fish and Wildlife Service and others in studies identifying the sources and causes of reproductive failure in birds and other wildlife in the Great Lakes region (Giesy et al. 1994; Hoffman et al. 1987; Jones et al. 1993, 1994; Newsted et al. 1995; Ratner et al. 1994; Tillitt et al. 1991, 1993; Williams et al. 1995). Advances in transgenic research have recently led to the production of recombinant cell lines in which reporter genes have been inserted downstream from DREs in the DNA of human, rodent, fish, and yeast cells (Anderson et al. 1995, 1996; El-Fouly et al. 1994; Garrison et al. 1996; Richter et al. 1997; Sanderson et al. 1996). In these cells, bacterial genes that code for firefly luciferase or bacterial β-galactosidase are activated by the AhR-dioxin complex, and the gene product that is formed causes the production of light or a color change that can be measured quantitatively. Advantages in sensitivity, selectivity,

ecological relevance, or ease of performance of the assay are claimed for these cell lines as compared with the H4IIE cell line. These recombinant cell lines will be evaluated and compared under sponsorship of the Dredging Operations Environmental Research Program, and the results will be published in future technical notes. In this note, the results are reported of the first year of work using the H4IIE cell line.

METHODS: Corps of Engineers (CE) District personnel were asked to provide subsamples of dredged sediments undergoing analysis for dioxins. Samples were received at irregular intervals from several coastal and Great Lakes CE Districts. On receipt at WES, the sediment samples were extracted according to U.S. Environmental Protection Agency (EPA) Method 3540 using Soxhlet apparatus initially and later using a DIONEX Accelerated Solvent Extraction (ASE™) apparatus. The extracts were cleaned on sulfuric acid-silica gel and were solvent-exchanged to isooctane. Assays were then performed using the Reuber H4IIE rat hepatoma cell line standardized against 2,3,7,8-TCDD (Appendix A). Results were expressed as TCDD TEQs and compared with the TCDD TEQs calculated for the analytical chemistry (GC/MS) results provided by the CE Districts on the same sediment samples.

RESULTS AND DISCUSSION: The H4IIE assay has been shown to have good reproducibility among laboratories and repeatability within a laboratory (Tillitt, Giesy, and Ankley 1991). Previously, the assay was performed using petri dishes and a separate protein determination. As with other enzyme assays, protein measurements are used to normalize the rate of formation of the product. The protocol given in Appendix A incorporates the advances developed by Sanderson et al. (1996) and is performed in 96-well microtitre plates (Figure 1), allowing much more rapid screening of environmental samples than is possible using petri dishes. Performance is also enhanced by the use of a fluorescence method for the protein determination, allowing resorufin and protein to be quantitated simultaneously on the same plate. Sensitivity of the assay is improved in the 96-well plate format and is quite high. The detection limit for the H4IIE assay performed in petri dishes was reported to be 10 pg per plate TCDD TEQ (Tillitt, Geisy, and Ankley 1991) and in the present study was 0.08 pg per well TCDD TEQ (equivalent to ~1.6 pg g^{-1}).

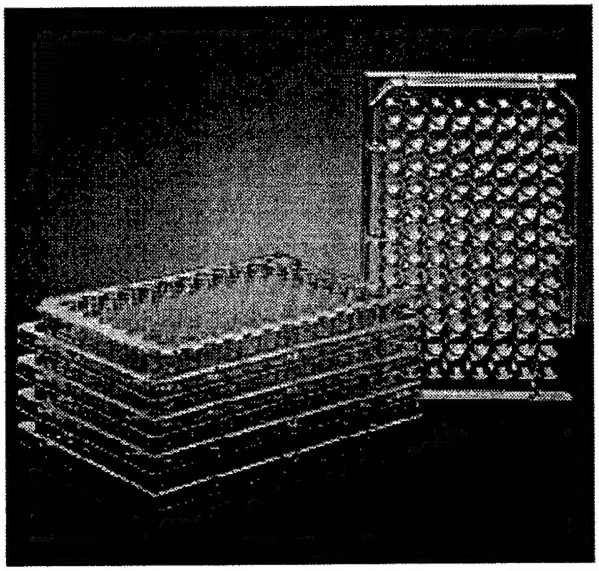

Figure 1. 96-well microtitre plates

Sample preparation for the cell-based assay requires an extraction step similar to that used for GC/MS analysis. Extractions are now performed using a DIONEX ASE™ system. The ASE complies with EPA Method 3545 for chlorinated hydrocarbons and for PAHs. The system uses liquid solvents at elevated temperature and pressure for extraction of solid samples. Samples are extracted rapidly with small volumes of solvent as compared with conventional methods. For

example, a 10-g sample can be extracted in about 15 min using 15 mL of solvent as compared with several hundred milliliters of solvent and many hours using Soxhlet apparatus.

As a check on performance of the ASE system, a sediment sample from Saginaw Bay, MI, was split and extracted using Soxhlet and using ASE. The solvent used was dichloromethane (DCM), and the samples were cleaned on sulfuric acid-silica gel to remove PAHs. The cleaned extracts were solvent-exchanged to isooctane and assayed using the H4IIE cell line standardized against 2,3,7,8-TCDD. Six replicates of each extraction were made, and the mean TCDD TEQs and SDs were 215 (77) pg g^{-1} for ASE and 202 (84) pg g^{-1} for Soxhlet extraction. These concentrations were not significantly different (P = 0.7856) by t-test.

Results obtained using the H4IIE dioxin screening assay were compared with the results of GC/MS analysis on 32 sediment samples. The screening assay returned results that were statistically not different from GC/MS results (Mann-Whitney Rank Sum Test, H4IIE median TEQ = 112, GC/MS median TEQ = 74, P = 0.059). However, when the log data were regressed (Figure 2), nearly one-half the values fell outside the 95-percent confidence interval. The variability has several sources. First, the screening assay and the GC/MS analyses were not performed on the same sample extracts, and there is always an appreciable heterogeneity in subsamples of even well-mixed soil or sediment samples. Additional variability is due to the fact that although most of the H4IIE results were the means of six replicates, the GC/MS analyses were not replicated. Also, the data set is dominated by sediments from a single source, Saginaw Bay, MI, and tended to cluster around a narrow concentration range. A better regression would result from a wider range of concentrations.

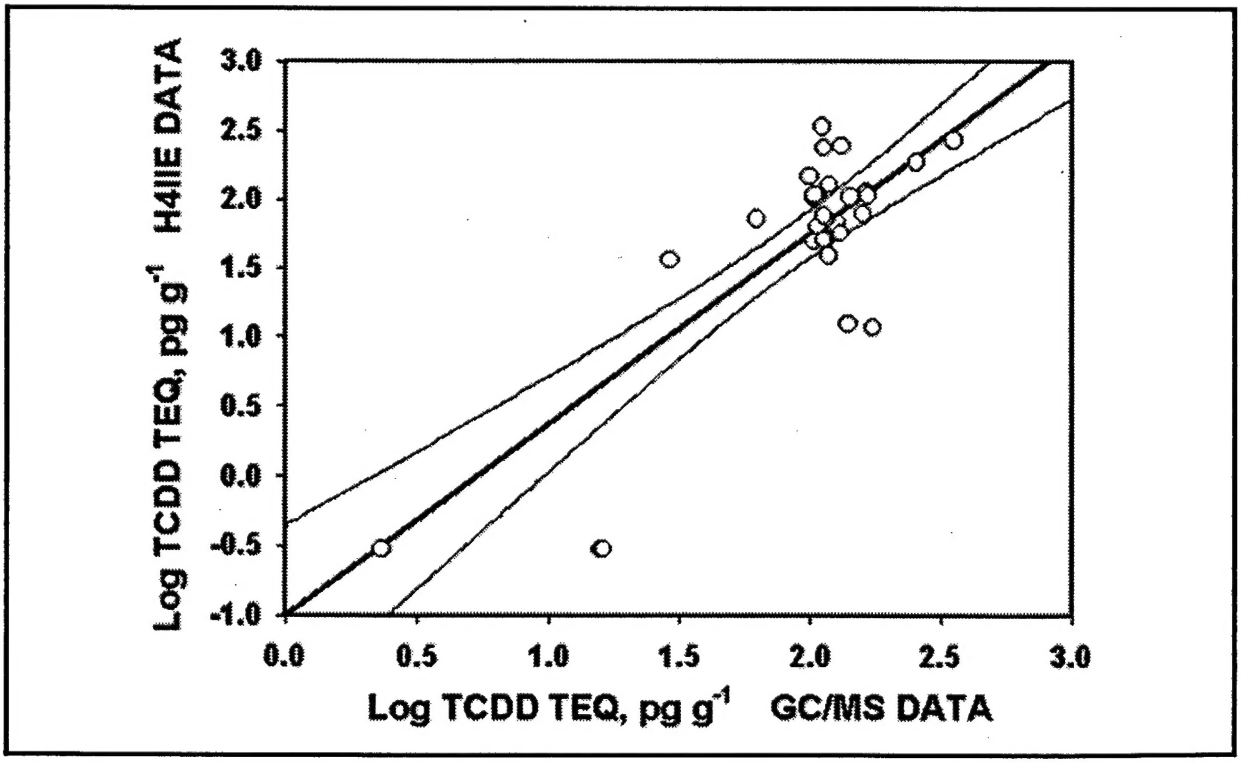

Figure 2. Dioxin TEQ measured by the H4IIE assay versus TEQ calculated for GC/MS results using I-TEFs on subsamples of the same sediments. Regression estimate and 95-percent confidence interval on the logarithms: $y = 1.377x - 1.00$, $r^2 = 0.706$

One-to-one correspondence between the two methods would not be expected, in any case. The reason is that the cells respond to all chemicals present that bind with the AhR, not just the 17 dioxin and dibenzofuran congeners that are analyzed by GC/MS. Even after cleanup to remove PAHs, there will typically be some non-dioxin/furan AhR-active compounds present, e.g., the non-ortho and mono-ortho PCBs. This fact actually enhances the toxicological relevance of the cell-based assay since AhR activity correlates directly with dioxin toxicity (Safe 1987, 1989, 1990, 1992; Safe et al. 1987). In any case, the variability observed is similar to that reported in other studies. The coefficient of variation (CV) for TCDD TEQs calculated for the Saginaw sediment was 35.8 percent for the ASE extracts and 41.6 percent for the Soxhlet extracts. These values are comparable with the average TCDD ED_{50} CV (33.8 percent) across bioassays reported by Tillitt, Giesy, and Ankley (1991) and with those reported by Clemons et al. (1996) for pure chemical standards (range: 11.8 to 72.0 percent).

Eight 96-well microtitre plates are a workable number for one technician to manage when conducting the H4IIE assay. Two of these plates are required for the standards. One plate is for the TCDD standards and one for the combined resorufin and protein standards. This permits a total of six plates to be used for the analysis of environmental samples; two samples, or two replicates of a sample, can be assayed on each plate. The total time required for preparation of a sediment extract was 16 hr over a period of 1 week using ASE. Most of the labor time was spent performing the silica gel cleanup step. The time required for performance of the H4IIE assay after extraction and cleanup was 11 hr. In this developmental work, two sediment samples, with six replicates per sample, were assayed over a period of 4 days. Computation of data required an additional 1.5 hr. Depending on user requirements, more samples (with fewer replicates) could be assayed in one eight-plate batch. If samples were not replicated and not cleaned to remove any PAHs that might be present, 12 sediments could be assayed at one time; the throughput (from extraction to computation) would reduce to about 5 hr per sample, or about $125/sample assuming a total cost for labor, supplies, and overhead of $25/hour. In situations involving the necessity of analyzing large numbers of samples, such as monitoring remediation effectiveness in the field, nonreplicated assays could provide acceptable resolution. Used as a screening assay, the cell-based method is a cost-effective means of prioritizing samples for more definitive GC/MS analyses.

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> U.S. Army Engineer Waterways Experiment Station. (1998). "Guidance for performance of the H4IIE dioxin screening assay," Technical Note DOER-C1, Vicksburg, MS.

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APPENDIX I
LABORATORY PROTOCOLS
FOR THE H4IIE DIOXIN SCREENING ASSAY

Protocol I. Cultures and Subcultures of Cells.

I. MATERIALS AND EQUIPMENT.

Laminar flow hood.

CO_2 incubator.

Ultrapure water still.

Sterile, disposable 5-mL, 10-mL, and Pasteur pipettes.

Small (e.g., 25-mL) beaker of 70-percent alcohol and forceps.

Sterile T-75 and T-25 culture flasks.

Water bath, 37 °C.

Aspirator.

Filter flask, 0.22-µm filters, and vacuum pump.

Rechargeable portable pipette and tips.

Inverted microscope.

50-mL test tube rack.

Cells obtained from American Type Culture Collection: ATTC CRL 1548. H-4-II-E (Hepatoma, Reuber, H35, rat).

Liquid N_2 Dewar, -180 °C.

II. REAGENTS.

A. FROM GIBCO BRL PRODUCTS.

Dulbecco's Modified Eagle Medium (DMEM) Cat. No. 10316024.

MEM Amino Acids Solution Cat. No. 11130-051.

MEM Non-Essential Amino Acids Solution Cat. No. 11140-019.

MEM Vitamin Solution Cat. No. 11120-052.

Fetal Bovine Serum (FBS) Cat. No. 16000-044.

L-Glutamine-200 mm Cat. No. 25030-016.

Dulbecco's Phosphate-Buffered Saline (PBS) Cat. No. 14190144.

Reagents will be aliquoted in bulk ahead of time and stored in refrigerator or freezer. Protocol II (Performance of the Assay) will indicate how many aliquots of each reagent will be needed. At least 20 aliquots of each reagent need to be prepared. Centrifuge tubes will be labeled with reagent, date, and initials of preparer.

B. PREPARATION.

1) **Dulbelco's Modified Eagle Media (DMEM)**, supplemented with vitamins, essential amino acids, nonessential amino acids, and L-glutamine, stored frozen.

a) Remove DMEM, vitamins (100x), essential amino acids (50x), nonessential amino acids (100x), and L-glutamine (100x) from refrigerator or freezer and place in 37 °C water bath.

b) When thawed, dry outside of containers with paper towel and wipe with paper towel moistened with 70-percent alcohol.

c) Aseptically aliquot the following components into sterile 50-mL centrifuge tubes:

Component	Volume mL
Vitamins (100x)	0.4
Essential amino acids (50x)	0.8
Nonessential amino acids (100x)	0.4
DMEM	34

d) Add 0.4 mL of L-glutamine (100x) to 1.0-mL cryovials separately from other media ingredients. Cap tubes tightly and store in test tube rack in freezer.

e) At the time of use, thaw DMEM mixture and L-glutamine vials and add the L-glutamine to the DMEM.

2) **Phosphate-Buffered Saline (PBS)**, 1x, stored refrigerated.

a) Aseptically prepare aliquots of 1x PBS by adding 4 mL of 10x PBS (stored at room temperature) and 36 mL sterile water to sterile 50-mL centrifuge tubes.

b) To prepare sterile water, aseptically filter 1 L Type I ultrapure water through a 0.22-µm filter flask and store refrigerated.

c) Cap tubes tightly and store in test tube rack in refrigerator.

3) **Fetal Bovine Serum (FBS)**, stored frozen.

a) Remove FBS from freezer and place in 37 °C water bath.

b) When thawed, dry outside of container with paper towel and wipe with paper towel moistened with 70-percent alcohol.

c) Aseptically aliquot 4.5 mL FBS into sterile 15-mL centrifuge tubes.

d) Cap tubes tightly and store in test tube rack in freezer.

4) **Trypsin**, 1x, stored frozen.

a) Remove trypsin (10x) from freezer and place in 37 °C water bath.

b) When thawed, dry outside of container with paper towel and wipe with paper towel moistened with 70-percent alcohol.

c) Aseptically prepare 5-mL aliquots of 1x trypsin.

i. To prepare 5 mL 1x trypsin.
In conical 15-mL capped centrifuge tube:
Add 0.5 mL sterile 10x PBS.
Add 4 mL sterile water.
Add 0.5 mL 10x frozen trypsin.

ii. Cap tubes tightly and store in test tube rack in freezer.

III. THE CELL CULTURE.

American Type Culture Collection (ATCC) CRL 158 H-4-II-E (Hepatoma, Reuber H35, rat) is a continuous cell line shipped frozen on dry ice. The H4IIE cells are frozen at a variable passage number (number of times primary culture was subcultured) by ATCC. However, upon receipt and initial culture, cells are cataloged "passage 0" and usage terminated at or near "passage 30" (approximately 15 TEQ assays) as an in-house quality control procedure. Two T-75 flasks are inoculated from one vial of H4IIE cells and are subcultured several times to obtain a total of 72 1-mL vial aliquots at approximately 10^6 cells/mL for frozen permanents.

A. STARTING THE CULTURE.

1) Wear lab jacket and powder-free gloves (nonsterile is fine) when handling cells. Sanitize gloves by squirting a small amount of 70-percent alcohol on gloves and rubbing hands until alcohol evaporates.

2) Thaw and culture frozen cells as soon as possible upon receipt. If cells cannot be thawed and cultured immediately upon receipt, store in liquid N_2 Dewar at -180 °C to prevent the loss of their viability. This method is also recommended for long-term storage.

3) *Warning: A protective full face mask, cryogenic gloves, and lab coat must be worn during storage or retrieval of cell vial in or from liquid N_2 Dewar.*

4) Allow vial to thaw by placing in 37 °C water bath. As soon as ice has melted (40-60 sec), remove vial from water bath and sanitize by squirting entire vial with 70-percent alcohol. Wipe the vial dry and store under sanitized laminar flow hood. *Note!* From this point on, sterile technique must be employed.

5) Mix contents of vial gently 2 to 3 times by aspirating with sterile 1-mL graduated pipette. Place one-half contents of vial into bottoms (back walls of flasks standing on end) of two T-75 flasks containing 10 mL DMEM each. This 10-fold dilution of the vial contents lowers the cryoprotective agent (DMSO) in which the cells were shipped to a concentration that does not necessitate its complete removal. In 24 hr, change the media (see Section IV, CHANGING THE MEDIA).

6) Check the H4IIE cells for confluency by observing with inverted microscope using 20x objective and Phase 3 sliding annulus. Confluent cells are "jammed wall-to-wall," with no, or little, room for the cells to divide.

a) Remove culture flasks one or two at a time from CO_2 incubator by first tightening the lids of the flasks *without lifting the flasks.*

b) Tilt the culture flask(s) back to prevent sloshing of the media into the neck of the flask. Be sure to keep inner glass door and outer door of CO_2 incubator closed as much as possible.

7) Check for visible signs of contamination, which include turbidity of media, floating objects in media, objects visible to the eye adhering to bottom of flask, and microscopic objects of a different morphology than the cells.

8) If cells are not confluent, proceed to Section IV, CHANGING THE MEDIA. If cells are confluent, proceed to Section V, SUBCULTURE OF CELLS.

IV. CHANGING THE MEDIA.

A. MEDIA PREPARATION.

1) Place aliquots of PBS (stored in 50-mL tubes) and FBS (stored in 15-mL tubes) into 37 °C water bath. One aliquot will be sufficient for up to 4 T-75 flasks or up to 10 T-25 flasks. Use one aliquot of FBS for each aliquot of media. The choice of T-150, T-75, or T-25 flasks is based on intended use. For instance, one confluent T-25 flask will suffice to set up seven 96-well plates with enough remaining to start a subculture.

2) When aliquots are thawed, remove from water bath, dry them with a paper towel, and wipe the outside of the tubes with 70-percent alcohol. Place the tubes in a tube rack under the hood and loosen the tube caps.

3) Triturate FBS by aspirating and expelling twice using a sterile 5-mL pipette tip and rechargeable pipette. Be careful not to aspirate the liquid too far up into the pipette tip. Prepare DMEM media by aseptically transferring 4 mL FBS into media tubes, bringing the total volume to 40 mL, at 10-percent FBS. Add 0.4 mL L-glutamine to the media. Triturate media several times using the sterile pipette. Replace 50-mL centrifuge tube cap, but do not tighten.

B. CELL PREPARATION.

1) Place up to four flasks (any size) in hood. Use care when transporting the flasks to avoid sloshing of media into the neck of the flask.

2) Stand flasks on end and loosen caps.

3) Place aspirator hose within easy reach, and remove forceps from alcohol. Wave forceps to evaporate alcohol. Be sure to keep forceps in the hood.

4) Remove top of Pasteur pipette autoclave box and remove a Pasteur pipette using sterile forceps, taking care to touch only one Pasteur pipette.

5) Touching only the extreme top of the Pasteur pipette (the part that is sure not to be placed into a flask), attach the aspirator hose to the Pasteur pipette and turn the vacuum pump on.

6) Remove one culture flask top at a time and place the tip of the Pasteur pipette into the media to aspirate the media out of the flask. Take care not to touch the outside or the neck of the flask with the Pasteur pipette. Be sure to lay tops on hood surface with open part of top facing up.

C. WASHING THE CELLS.

1) Using a 5- or 10-mL sterile pipette, pipette 3 to 4 mL PBS into the culture flasks and lay the flasks on their side to wash the cells with the PBS.

2) Set the flasks back up on end and aspirate the PBS with a sterile Pasteur pipette as in Steps B 2 through 5.

D. ADDING FRESH MEDIA.

1) Using a 5- or 10-mL sterile pipette, add 10 mL of fresh media to each T-75 flask or 4-mL fresh media to each T-25 flask.

2) Replace culture flask tops and tighten. Lay flasks on side to cover cells.

3) Aspirate leftover media and FBS for disposal, remove items from hood, and wipe down hood with 70-percent alcohol. Proceed to Protocol II.

V. SUBCULTURE OF CELLS.

First determine the number of flasks needed. The purpose of subculturing cells is to dilute the cell density, giving space for the cells to grow. All of the cells from the confluent flask may be kept and cultured in new flasks, or a portion of the cells may be discarded just to maintain the cell culture. If large numbers of cells are desired, subculture up to four flasks from one confluent flask (either size). If maintenance levels of cells are desired, subculture one flask from one confluent flask, discarding the majority of the cells.

A. FLASK PREPARATION.

1) Place appropriate size flasks (still in plastic bags) under the hood and remove the number of flasks needed to subculture cells.

2) Tape bags while still under hood and replace in drawer.

3) Label the flasks with cell type, passage no. (add 1 to the passage no. of the flask from which you are subculturing), date, and your initials.

4) Stand flasks on end and loosen caps.

B. MEDIA PREPARATION.

1) Refer to Sections IV A - D for media preparation, preparing the culture flasks, and washing the cells.

2) Using a sterile 10-mL pipette, pipette 9 mL of media into each new T-75 flask or 3 mL media into each new T-25 flask. Replace caps and place flasks in back of hood out of the way.

C. TRYPSINIZING THE CELLS.

The cells secrete a protein that causes them to adhere to the culture flask. Trypsin is an enzyme that hydrolyzes this protein. FBS (which is a component of the prepared media) contains Trypsin Inhibitor, a substance that stops the action of trypsin. The entire trypsinization process should not last more than 10 min, or the cells may be damaged.

1) Using a 5- or 10-mL sterile pipette, pipette 4 mL trypsin into each T-75 (or 2 mL into each T-25) flask and lay the flasks on their side to coat the cells with the trypsin. Tighten the caps and place in the CO_2 incubator. Remember to carry the flasks tilted backward. Do not loosen the caps. Allow the flasks to incubate 3 to 4 min.

2) Remove a flask and gently but firmly tap the side of the flask 4 to 5 times against a bench top edge to loosen the cells. Observe the flask under the inverted microscope to see if the cells are loosened. If they are not, replace the flask in the CO_2 incubator for another 1 to 2 min and repeat the procedure. If the cells are loose, remove the remaining flasks from the incubator and tap them also.

3) Transport the flasks back to the hood, set them on end, and loosen the caps. Add one drop of FBS to stop the trypsin action.

D. LOADING NEW CELLS.

1) Refer to Sections IV A - D for media preparation, preparing the culture flasks, and washing the cells.

2) Using a 5-mL sterile pipette, triturate the cells to break up the clumps, and pipette a portion of the cell suspension into each of the new flasks already containing fresh media. Pipette 1 mL into T-75 flasks and 0.2 mL into T-25 flasks.

3) Replace culture flask tops and tighten. Lay flasks on side and gently shake to cover the flask with media.

4) Place flasks on side in CO_2 incubator.

5) Aspirate leftover media and FBS for disposal, remove items from hood, and wipe down hood with 70-percent alcohol.

Protocol II. Performance of the Assay.

I. PRELIMINARY.

Four days are required for performance of the assay. The total time required will depend to a large extent on the method used to extract the sediment sample, whether or not a cleanup step is included, and the number of replicates per sample. Sediment extraction and cleanup protocols are not included in this document, but procedures that are appropriate for GC/MS analysis, e.g., EPA Method 3540, are appropriate for cell-based assays after solvent exchange to isooctane. On Day 0 (preferably a Monday or a Thursday), cells are plated in 96-well microtitre plates and incubated overnight to allow adherence of the cells to the plate. On Day 1, the cells are spiked either with environmental extracts or 2,3,7,8-TCDD standards and are incubated for 72 hr to allow time for enzyme induction. On Day 4, the cells are lysed and cytochrome P450 (specifically ethoxyresorufin O-deethylase (EROD)) activity is quantitated using an enzyme reaction, the O-deethylation of ethoxyresorufin, to form a fluorescent product, resorufin. The formed resorufin is quantitated using a fluorescence microplate reader.

For each assay, one 96-well plate will be needed for TCDD standards, and one 96-well plate will be needed for every two environmental samples. Outer wells of 96-well plates are not used because the cells do not culture well in them, resulting in 60 available wells per plate. For the TCDD standard, 5 replicates are made for six of its nine concentrations (30 wells), and 10 replicates are made for the remaining three (30 wells). Similarly, environmental samples have five replicates of each of their six concentrations (first environmental sample plate has five concentrations because first row is used as negative control with 10 replicates). On Day 4 of the assay, another plate will need to be set up to generate a resorufin standard curve and a protein standard curve.

Time required to complete each daily procedure using eight 96-well plates (one TCDD standard plate, one combined resorufin and protein standard plate, and six plates for environmental samples) is described below:

	Time, hr
Day 0	3.0
Day 1	2.5
Day 4	3.5
Solution Preparation	2.0
Total	11.0

II. Day 0.

A. MATERIALS AND EQUIPMENT.

DMEM - 15 mL for each 96-well plate using 60 wells.

FBS - one aliquot for each aliquot of DMEM.

Glutamine - one aliquot for each aliquot of DMEM.

Trypsin - one aliquot for each T-25 flask to be trypsinized.

PBS - one aliquot.

Electronic 8 Channel Motorized Pipette, 250 µL.

Sterile 250-µL Rainin pipette tips.

Water bath set at 37 °C.

Hemacytometer and coverslip.

Inverted microscope.

B. PROCEDURE (Use sterile technique).

1) Turn laminar flow hood on and wipe down with 70-percent alcohol. Allow at least 15 min for equilibration. Turn on 37 °C water bath.

2) Determine the number of 96-well plates needed.

3) Remove sufficient DMEM, FBS, and glutamine from freezer. Place DMEM, FBS, trypsin, and PBS in water bath to thaw. Let glutamine thaw at room temperature.

4) Prepare DMEM and trypsinize one confluent T-25 flask (Protocol I) for every seven 96-well plates to be used.

5) Count the cells in the trypsinized flask using the hemacytometer to determine the number of cells/mL of the culture stock.

6) In the 96-well plates, plate 7,300 cells/well in 250 µL DMEM per well. Use the following formula to calculate the cell concentration needed to plate the cells:

   a) Given 7,300 cells/well, 7,300 cells/0.250 mL = 29,200 cells/mL, and given 60 wells × 0.250 mL/well = 15 mL (round up to 20 mL to have extra), make 20 mL of DMEM containing 29,200 cells/mL for each 96-well plate. For example, to make one plate: (29,200 cells/mL)(20 mL) = (stock cells/mL) (× mL).

7) Incubate overnight in CO_2 incubator.

III. DAY 1.

The cells are dosed with either a 2,3,7,8-TCDD standard or environmental sample today. The standard and sample preparation does not have to be carried out aseptically. Isooctane will not support the growth of microorganisms, so any microbes introduced to the cells via the solvent will be dead. When dosing the cells, however, use sterile pipette tips and work under the laminar flow hood. The pipette tips will come in contact with the media and the 96-well plate. Removal of the 96-well plate cover should always be performed under the laminar flow hood. Be sure to dose the cells at a convenient time. The EROD assay will be performed 72 hr after dosing, so allow time before and after the 72-hr mark for preparation work and for the assay.

A. MATERIALS.

TCDD standard - 1.0 µg/mL in isooctane, stored in freezer.

Extract of environmental sample (ES) in isooctane, also in freezer.

Isooctane, pesticide grade.

Electronic 8 Channel Motorized Pipette, 10 µL.

Sterile 10-µL pipette tips.

Electronic Motorized Pipette, 100 µL and 1,000 µL.

250-µL and 1,000-µL pipette tips.

8-well strips.

50-mL solution basins (to use as reagent reservoirs).

Microcentrifuge tubes.

Ultraviolet (UV) degrader/acetone.

B. PROCEDURE

IMPORTANT! 2,3,7,8-TCDD is highly toxic. Wear latex gloves, lab coat, and glasses while handling TCDD. Work under fume hood and do not spill any!! Understand this section thoroughly before beginning.

1) Turn on laminar flow hood and wipe down with 70-percent alcohol. Allow at least 15 min for equilibration.

2) Remove TCDD standard and ES extract from freezer and allow to come to room temperature.

3) Prepare TCDD working stock.

   a) Vortex 15-mL amber vial TCDD 1.0-µg/mL stock standard after reaching room temperature. Aliquot 500 µL of stock into 1-mL Teflon cap vial prelabeled "1.0-µg/mL TCDD" for a working stock. Return main stock to freezer with Teflon cap wrapped in parafilm. Working stock will be diluted 3x (1:10 twice and 1:5 once) to obtain highest dosing concentrations.

   b) Place 90-µL isooctane into two microcentrifuge tubes and 160 µL into a third one.

   c) Pipette 10 µL of the 1.0-µg/mL TCDD working stock into the first tube containing the 90 µL isooctane (creating a 0.1-µg/mL or 100-ng/mL TCDD solution). Mix the solution with the pipette.

   d) Pipette 10 µL of the solution from the first tube into the second tube containing 90 µL isooctane (creating a 10-ng/mL TCDD solution). Mix the solution with the pipette.

   e) Pipette 40 µL of the solution from the second into the third tube containing 160 µL isooctane (creating a 2.0-ng/mL TCDD solution and beginning dose). Mix the solution with the pipette.

4) Prepare TCDD serial dilutions for 2.0 to 0.156 ng/mL (10.0 - 0.0781 pg/well).

   a) Take two 8-well strips and pipette 50 µL of isooctane into 2nd through 8th well of the first strip (Strip A) and the first well of the second strip (Strip B).

b) Pipette 50 µL of the solution from the third microcentrifuge tube (from Step 3e) into the 1st and 2nd wells of Strip A, creating 100 µL of a 1.0-ng/mL TCDD solution in the 2nd well. Mix the solution using the pipette.

c) Pipette 50 µL of the 1-ng/mL TCDD solution into the 7th well (creating 100 µL of a 0.5-ng/mL TCDD solution) and mix.

d) Repeat Step c for the 8th of Strip A and 1st well of Strip B, serial diluting to generate the 0.125-ng/mL TCDD solution in the 1st well of Strip B.

e) Serial dilute the 0.125-ng/mL TCDD standard by pipetting 50 µL from 1st well of Strip B to 3rd well of Strip A and mix (creating 100 µL of a 0.0625-ng/mL TCDD solution).

f) Continue serial dilutions for Wells 4 and 5 of Strip A, generating the 0.03158- and 0.015625-ng/mL TCDD solutions. The 6th well will only have isooctane for solvent control.

g) Wells 1 through 6 of Strip A will be utilized for dosing to obtain the 10.0- to 0.0781-pg/well standards. Dose the cells immediately after preparing the standards to prevent loss of solvent due to volatilization (see Step 6).

5) Prepare TCDD serial dilutions for 1.0 to 0.0625 ng/mL (5.0 to 0.3125 pg/well).

a) Take one 8-well strip and pipette 50 µL of isooctane into the 2nd through 7th wells.

b) Pipette 50 µL of the solution from the third microcentrifuge tube (from Step 3e) into the 2nd well, creating 100 µL of a 1.0-ng/mL TCDD solution. Mix the solution using the pipette.

c) Pipette 50 µL of this solution into the 3rd well and mix, creating 100 µL of a 0.5-ng/mL TCDD solution.

d) Repeat Step c for the 4th through 6th wells of the 8-well strip, serially diluting the TCDD 1:2 and leaving the 7th well for a solvent control. Wells 2 through 7 are utilized for dosing to generate the 5- to 0.3125-pg/well TCDD standards. Dose the cells immediately after preparing the standards to prevent loss of solvent due to volatilization (see Step 6).

6) Using the Electronic 8-channel pipette without pipette tips on positions 1 and 8, pipette 5 µL of each solution into all the cells of a row, as illustrated in the schematic below. Dose the cells in the laminar flow hood using sterile technique *immediately* after making each 8-well strip of standard.

7) Prepare the environmental sample dilutions for dosing. Five replicates of six dilutions of the extracts are run in the assay. Prepare one 8-well strip at a time and *dose immediately* to prevent volatilization of the isooctane. *Note:* First two environmental samples will have only five dilutions because negative controls (n = 10) are run in first set of wells.

Schematic of TCDD Standard Curve Plate (Values are pg/well)											
	solvent control	solvent control	solvent control	solvent control	solvent control	solvent control	solvent control	solvent control	solvent control	solvent control	
	TCDD 0.0781	→				TCDD 0.3125	→				
	TCDD 0.1561	→				TCDD 0.625	→				
	TCDD 0.3125	→				TCDD 1.25	→				
	TCDD 5.0	→				TCDD 2.5	→				
	TCDD 10.00	→				TCDD 5.0	→				

a) Use an 8-well strip to make the dilutions.

b) Pipette 50 µL isooctane into 3rd through 6th wells of the 8-well strips used in dosing the first set of environmental samples. All remaining environmental samples will have 50 µL in 2nd through 6th of the 8-well strips.

c) Illustrated below is an 8-well setup for first set of environmental samples (skip the 1st, 2nd ,7th , and 8th well of the 8-well strip):

empty	empty	50 µL isooct	50 µL isooct	50 µL isooct	50 µL isooct	empty	empty

d) Pipette 50 µL of environmental sample 1 into 2nd and 3rd well. Mix by aspirating and dispensing solution with pipette.

e) Transfer 50 µL of the sample in 3rd well to the 4th well and mix.

f) Transfer 50 µL of the solution in 4th well to the 5th well and mix.

g) Repeat Step f for solution in 5th to 6th and mix. *Note:* Go to Step 8 at this point for first two environmental samples or continue on to Step h for remaining environmental samples.

h) Repeat Step f for solution in 6th to 7th and mix.

i) Dose cells (see Step 8).

j) Use one 8-well strip and repeat Steps c - i for each sample.

8) Dose the cells for first set of environmental samples in the laminar flow hood following the schematic below without using outer wells. Use sterile technique:

Schematic of first environmental sample (ES) plate w/dilution number (i.e., D1 lowest dilution and D5 highest dilution) n = 5 and negative (NG) control n = 10

	NG control	NG control	NG control	NG control	NG control	NG control	NG control	NG control	NG control	NG control	
	ES1 D1	→				ES2 D1	→				
	ES1 D2	→				ES2 D2	→				
	ES1 D3	→				ES2 D3	→				
	ES1 D4	→				ES2 D4	→				
	ES1 D5	→				ES2 D5	→				

9) Transfer remaining solutions of TCDD standard and environmental sample solutions into the UV degrader and add 10 to 20 volumes of acetone. Turn the UV degrader on for 72 hr to degrade the TCDD.

10) Return TCDD working stock and environmental samples to freezer.

IV. DAY 4.

The cells have been incubating with either TCDD standards or environmental samples for 3 days. This allows time for maximal enzyme (EROD) induction. The assay should be performed at *as close as possible to 72 hr after dosing of the cells.* Prepare the needed solutions (HEPES buffer, dicumarol solution, ethoxyresorufin solution, etc.) *before* the 72-hr mark. Calculate how much of each solution will be needed and make extra.

Today, an additional plate will be needed to make the protein standard curve. Half of the plate (five columns) will be used for the resorufin curve, and the other half will be used for the protein curve. Prepare the resorufin and protein standard plate (Standard Plate) either well before or after the 72-hr mark for the incubating cells. Fluorescamine will be added to each well to stop EROD induction. The fluorescamine binds to protein and fluoresces, allowing quantitation of the protein in the sample.

A. MATERIALS.

1) Stock solutions to be prepared ahead of time:

   a) **1x Phosphate Buffer**. See Protocol I, Reagents.

   b) **50-mm HEPES Buffer Stock**. Dissolve 6.0 g HEPES in 500-mL ultrapure water and adjust to 7.8 pH with drops of concentrated HCL. Store in refrigerator.

c) **100-µm (24.2-µg/mL) Ethoxyresorufin (ER) Stock** . Dissolve 1 mg ER in 41.32 mL methanol. Store in 5-mL aliquots in amber vials in freezer to prevent degradation of stock solution due to repeated freezing and thawing.

d) **1-mm (240-µg/mL) Resorufin Stock**. Dissolve 24 mg in 100 mL methanol. Store in microcentrifuge tubes (200-µL aliquots) in freezer.

e) **Bovine Serum Albumin (BSA) Stock (5 mg/mL)**. Dissolve 50 mg BSA in 10 mL HEPES buffer. Store in refrigerator.

f) **1.08-mm Fluorescamine**. Dissolve 6 mg fluorescamine in 30 mL acetonitrile. Store in refrigerator. 30 mL are needed per assay (seven assay plates and one standard plate).

2) Solutions to be prepared on day of assay:

a) **HEPES buffer (pH 7.8)**. 100 mL are needed per assay including amount required for the following solutions.

b) **20-µm (4.8-µg/mL) Ethoxyresorufin Working Solution**. Dilute 100-µm ER stock 1:5 in HEPES buffer (1 part stock to 4 parts buffer). 25 mL are needed per assay. Make fresh and check absorbance of the 20 µm ER daily at 482 nm on Shimadzu UV160. Absorbance should be 0.450 ± 0.0225. Adjust ER solution by adding 100 µm ER stock or HEPES buffer as necessary until absorbance is correct.

c) **80-µm (26-µg/mL) Dicumarol**. Dissolve 1.3 mg Dicumarol in 50 mL HEPES buffer. Dicumarol is difficult to put into solution and will require use of a sonicator. Make fresh daily. 25 mL are needed per assay.

d) **0.5-mm (0.42-mg/mL) NADPH**. Dissolve 0.42 mg NADPH per mL HEPES buffer. Make fresh daily just prior to use. 25 mL are needed per assay. 10.5 mg NADPH will make 25 mL in HEPES.

3) Other materials and equipment:

a) Fluorescence Plate Reader.

b) 550-nm Excitation Filter and 590 Emission Filter for resorufin.

c) 400-nm Excitation Filter and 450 Emission Filter for protein/fluorescamine.

d) 96-well plate washer.

e) 8-well strips.

f) 13- by 100-mm culture tubes.

g) 50-mL solution basins.

B. PROCEDURE (Sterile technique not necessary).

1) Enzyme Assay.

a) Place about 500 mL 1x PBS in the wash solution and distilled water bottles of the plate washer. Make sure the waste bottle is empty.

b) Turn on both switches on the back of the 96-well plate washer to activate the instrument and the vacuum pump.

c) Place a purge plate in the washer and press the "Purge" touchpad.

d) Remove one 96-well plate from the CO_2 incubator and place in the 96-well plate washer. First a set of four, and then a set of three plates is processed at a time, allowing 10-15 min between each set.

e) Press the "Start" touchpad to wash the cells using microplate washer Protocol I (3x with PBS not sterile, the numbers 9 0 1 3 should be displayed on the plate washer).

f) Dispense 20 to 30 mL of ultrapure water into a reagent reservoir.

g) Pipette 30 µL ultrapure water in each well of the plate containing the cells.

h) Let sit 5 min at -80 °C to lyse cells; then thaw at room temperature for 10 min.

i) From reagent reservoir, pipette 100 µL of 40 µm dicumarol into each well of the plate.

j) From reagent reservoir, add 50 µL of the diluted ER to each well of the plate; then incubate at 37 °C for 20 min to acclimatize.

k) From reagent reservoir, add 50 µL NADPH to each well of the plate to start EROD reaction and return to incubator at 37 °C for 60 min. During the incubation time, standard resorufin and protein plates can be made.

l) Stop reaction with 50 µL fluorescamine; let stand 15 min at room temperature in dark area to avoid excitation due to fluorescent ceiling lights.

m) Read resorufin values in plate reader @ 550-nm excitation and 590-nm emission using a voltage setting of 2.8.

n) Save the data using the file format in Section V of Protocol II.

2) Standard Plate.

a) Turn on the computer attached to the Fluorescence plate reader.

b) Insert the 550-nm excitation filter and the 590-nm emission filter into the plate reader. Make sure they are in the right orientation.

c) Turn on the plate reader and adjust the voltage output to 2.8.

d) Pipette 30 µL ultrapure water into each well of the Standard Plate.

e) Pipette 200 µL of HEPES buffer into each well of the Standard Plate.

f) Prepare protein standards for standard curve.

   i. Obtain an 8-well strip.

   ii. Pipette 100 µL HEPES buffer into Wells 2 through 7.

   iii. Pipette 100 µL of the BSA Stock into the 2nd well (making 0.2 mL of 2.5 mg/mL) and mix with the pipette.

   iv. Pipette 100 µL of the solution in the 2nd well into the 3rd well (making 200 µL of 1.25 mg/mL) and mix with the pipette.

   v. Continue the serial dilution to the 6th well, making solutions of 2.5, 1.25, 0.625, 0.313, and 0.156 mg/mL (or 25, 12.5, 6.25, 3.13, and 1.56 µg/10 µL, respectively). Leave the 7th well for a blank (0 µg/10 µL).

g) Prepare resorufin standards for standard curve.

   i. Pipette 100 µL of the resorufin (RES) stock solution into a 13- by 100-mm culture tube and add 4.9 mL of the HEPES buffer. Check RES absorbance on Shimadzu UV160 at 572 nm. The correct absorbance is 0.920 ± 0.046 (3,000 ng/mL or 60 ng/20 µL). Adjust RES solution by adding drops of 1,000 µm RES stock or HEPES buffer. Calculate actual concentration by using Beer's Law: $A = \varepsilon_{RES}\, bc$, $\varepsilon_{RES} = 0.073\ l\ \mu Mol^{-1} * cm^{-1}$.

   ii. Obtain an 8-well strip.

   iii. Pipette 140 µL of HEPES Buffer into Wells 2 through 7.

   iv. Pipette 140 µL of the diluted resorufin stock into the 2nd well (making 280 µL of 1,500-ng/mL resorufin) and mix with the pipette.

   v. Pipette 140 µL of the solution in the 2nd well into the 3rd well (making 280 µL of 750-ng/mL resorufin) and mix with the pipette.

   vi. Continue the serial dilution to the 6th well, making solutions of 1,500, 750, 375, 187.5, and 93.75 ng/mL (or 30, 15, 7.5, 3.75, and 1.875 ng/20 µL, respectively). Leave the 7th well for a blank (0 ng/mL).

h) Pipette 20 µL of each resorufin and 10 µL of each protein standard in a 96-well plate as illustrated in the schematic below. Be sure the concentrations of the solutions are in descending order.

i) In the Biolinx software, set up the template to create a standard curve and recall the resorufin curve fit.

j) Place the Standard Plate in the plate reader and read @ 550-nm excitation and 590-nm emission using a voltage setting of 2.8.

Resorufin (20 µL)						Protein (10 µL)					
0 ng	→					0 µg	→				
1.88 ng	→					1.56 µg	→				
3.75 ng	→					3.13 µg	→				
7.5 ng	→					6.25 µg	→				
15 ng	→					12.5 µg	→				
30 ng	→					25 µg	→				

k) The standard curve generated should be linear, and the highest fluorescence values should be in the 2500 to 3700 range. If that is the case, record the A (slope) and B (intercept) values to later generate the equation $y = Ax + B$.

l) Save the data using the file format in Section VI, FILE LABELING FORMAT.

m) Remove plate from plate reader and place aside for protein determinations.

n) After all resorufin determinations, read the protein Standard Plate and add 50 µL 1.08 mm fluorescamine to each well and allow to sit 10 min.

o) In the analysis software, set up the template to create a standard curve and recall the protein curve fit.

p) Read in plate reader @ 400-nm excitation and 450-nm emission using a voltage setting of 5.0.

q) The standard curve generated should be linear, and the highest fluorescence values should be in the 1400 to 1800 range. If that is the case, record the A and B values to later generate the equation $y = Ax + B$.

r) Save the data using the file format in Section VI, FILE LABELING FORMAT.

s) Place waste material from 96-well plate washer containing DMEM, TCDD standard, and environmental sample solutions into the UV degrader and add 10 to 20 volumes acetone per one volume TCDD plus ES. Turn the UV degrader on for 72 hr.

V. DATA PROCESSING.

Due to the toxicity of the sediment extracts, it was not possible to calculate ED_{50}s as has been done using pure compounds. For that reason, the following method of standardization was employed. An alternative to the method given here is the slope ratio method (Ankley et al. 1991). The slope ratio method is currently being investigated to determine whether advantages exist in using it.

A. TCDD STANDARD CURVE GENERATION.

1) From the protein and resorufin standard plates data, generate a linear standard curve (µg protein or ng resorufin versus fluorescence) for both.

2) Determine the protein and resorufin values for the TCDD standard plate utilizing the linear standard curves generated in Step 1.

3) Calculate the ng resorufin/µg protein for each TCDD standard plate well. Eliminate any wells in which the protein values are 0.5 or 2 times the solvent control values (eliminates wells in which toxicity occurred).

4) Generate a log-linear TCDD standard curve (log pg TCDD/well versus ng resorufin/µg protein) utilizing only the points that are linear (the curve will be sigmoidal).

B. SAMPLE ANALYSIS.

1) Convert the sample raw data to ug protein and ng resorufin utilizing the standard curves generated in Step A1.

2) Calculate the ng resorufin/ug protein for each well.

3) Convert the pg TCDD equivalents/well utilizing the standard curve generated in Step A4. Average the values for each sample dilution.

4) For dilutions that give TCDD values at least 1.5 times the average background level, but less than the maximal response for the extract; correct for dilutions to obtain pg TCDD equivalents /g sample.

5) Average the values obtained from the dilutions for the final value.

6) Reporting nondetects: If no wells responded by at least 1.5 times the background level, but no wells indicated toxicity (protein level 0.5 background), the sample should be rerun after concentrating the extract. However, if at least one dilution indicated toxicity, then the sample can be recorded as below detection limits.

VI. FILE LABELING FORMAT.

A. Code:000XXX11.dat

1) 000 = Day of year, e.g. 001 is 1 Jan, 233 is 21 Aug.

2) XXX = Project ID, a three-letter designation recorded in the lab notebook.

3) 11 = Plate number analyzed that day. Use lab notebook to designate what samples are on plate and reading for whether resorufin or protein. ALWAYS make plates 1 - 4 the following:

   01 = TCDD Standard Resorufin Reading.

   02 = TCDD Standard Protein Reading.

   03 = Resorufin Standard Curve Reading.

   04 = Protein Standard Curve Reading.